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# Importance of Toll-Like Receptor 9 in Host Defense against M1T1 Group A *Streptococcus* Infections

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## Key Words

Toll-like receptor 9 · Group A *Streptococcus* · Innate immunity

## Abstract

Timely recognition and elimination of invasive group A *Streptococcus* (GAS) by innate immunity is crucial to control infection. The intracellular pattern recognition receptor Toll-like receptor 9 (TLR9) promotes macrophage hypoxia-inducible factor-1 $\alpha$  levels, oxidative burst and nitric oxide production in response to GAS. TLR9 contributes to GAS clearance in vivo in both localized cutaneous and systemic infection models.

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## Introduction

The Gram-positive bacterium group A *Streptococcus* (GAS) is a leading human pathogen causing >700 million superficial infections such as pharyngitis or pyoderma and >650,000 invasive infections worldwide every year

[1]. The host's initial defense mechanisms against GAS involve the physical barriers of mucosa or skin and their commensal microflora. Once GAS has breached the epithelium, eradication by soluble factors and phagocytes is required, and quick and accurate recognition of GAS by the host innate immune system can prevent disease spread. Pattern recognition receptors such as Toll-like receptors (TLRs) recognize conserved molecular patterns from pathogens; most TLRs will initiate signal transduction via the central adaptor protein myeloid differentiation factor 88 (MyD88). MyD88 has recently been shown to play an important role in innate defense against GAS infection [2].

Though classically described as an extracellular pathogen, studies have shown that GAS may survive intracellularly for several hours within epithelial cells [3–5], macrophages [6, 7] and neutrophils [8, 9]. These data led us to hypothesize that the intracellularly localized TLR9 could be important for GAS detection and elimination. TLR9 is found in endosomes and is stimulated by nonmethylated DNA and synthetic CpG oligodeoxynucleotides [10, 11] or during infection with obligate intracellular bacteria including *Listeria monocytogenes* and *Legionella pneu-*

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*mophila* [12, 13]. To date, the role of TLR9 in innate defense against bacteria that exist mainly extracellularly has not been fully elucidated [14, 15] and remains controversial [16–19].

## Methods

### *Mice and Bacterial Strains*

C57BL/6 wild-type (WT) and TLR9-deficient mice in the C57BL/6 background were used in this study. TLR9-deficient mice were a gift from Dr. Shizuo Akira (Osaka University, Japan) and were bred and handled in our facilities under the approved protocols of the University of California San Diego Animal Care Committee. WT mice were purchased from Charles River.

GAS MIT1 strain 5448 was originally isolated from a patient with necrotizing fasciitis and toxic shock syndrome [20]. GAS were propagated in Todd-Hewitt broth (Difco, BD Diagnostics) or Todd-Hewitt agar plates. In macrophage and mouse challenge studies, bacteria were grown to logarithmic phase in Todd-Hewitt broth [optical density at 600 nm = 0.4 = approx.  $2 \times 10^8$  colony-forming units (CFU)/ml], pelleted, washed and resuspended in PBS or in tissue culture media at the desired concentration.

### *Macrophage Killing Assays*

Peritoneal macrophages were isolated from the peritoneal cavity of WT and TLR9-deficient mice 72 h after injection of thioglycollate, as previously described [21]. Harvested macrophages were washed and resuspended in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS). In a 48-well plate,  $10^6$  macrophages were added per well and incubated for 24 h at 37°C with 5% CO<sub>2</sub>. Then cells were washed twice and resuspended in RPMI-1640 containing 2% FBS. Where indicated, the TLR9 antagonist guanosine-rich inhibitory oligonucleotide (G-ODN; 5 µM; Invivogen, Carlsbad, Calif., USA) was added. After 1 h, logarithmic phase bacteria were added to the wells at a final multiplicity of infection (MOI) of 0.1–0.2 and centrifuged for 5 min at 1,500 rpm. After 4 h of incubation at 37°C with 5% CO<sub>2</sub>, macrophages were detached with trypsin and lysed with 0.025% Triton X in PBS for serial dilutions to enumerate surviving bacteria (plated on Todd-Hewitt agar).

### *Oxidative Burst and Nitric Oxide Expression*

For reactive oxygen species (ROS) determination, bone marrow (BM)-derived macrophages were used. The BM cells of femurs and tibias of WT and TLR9-deficient mice were collected. Cells were plated in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS and 30% conditioned medium as described elsewhere [22]. Mature adherent BM-derived macrophages were harvested by gentle scraping after 7 days in culture and reseeded for the assay. On day 10, macrophages were washed twice, resuspended in Hanks' balanced salt solution and incubated with 30 µM 2',7'-dichlorofluorescein diacetate for 30 min of rotation at 37°C. Cells were washed 3 times with PBS and resuspended in Hanks' balanced salt solution for a final concentration of  $5 \times 10^5$  in 100 µl of solution per well of a 96-well plate. Where indicated, logarithmic phase bacteria were added at an MOI of 1:1. After 270 min, the fluorescence

signal was measured with excitation at 485 nm and emission at 535 nm using a fluorescent plate reader (Wallac II, Perkin Elmer). Nitric oxide (NO) expression was determined by the Griess reaction in peritoneal macrophages as previously described [23]; baseline values (no GAS stimulation) were subtracted from indicated values.

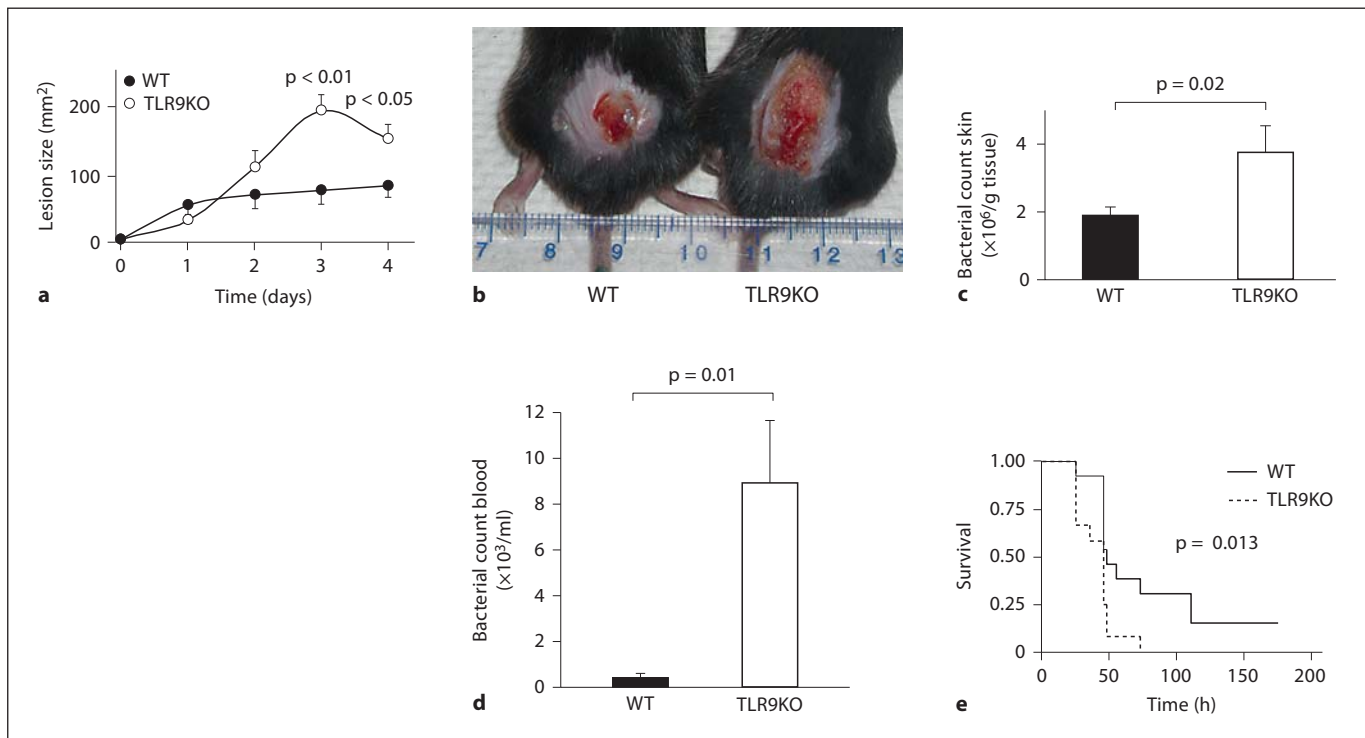
### *Hypoxia-Inducible Factor-1α Detection by Western Blot and Immunostaining*

BM-derived macrophages were harvested and cultured as described above. After 7 days in culture, mature macrophages were reseeded into 100-mm<sup>2</sup> wells for Western blot analysis or onto 12-mm<sup>2</sup> glass cover slides in 24-well plates for immunostaining. Macrophages were then either challenged with WT GAS (MOI 10) or were not exposed to bacteria. After 1 h of incubation, penicillin and gentamicin (10 and 100 µg/ml, respectively) were added to all the wells and macrophages were incubated for an additional 3 h. For Western blot analysis, cells were harvested and washed (in PBS), and proteins were extracted with RIPA buffer. Nuclear extracts (20 µg) were loaded onto a 12% Tris-tricine gel in a 2-(N-morpholino)ethanesulfonic acid buffer (Invitrogen) for hypoxia-inducible factor (HIF)-1α Western blot using standard methodologies [24]. For immunostaining, cells were immediately fixed with 4% paraformaldehyde and kept at 4°C until further analysis. After washing, cells were permeabilized for 45 min with PBS containing 2% BSA, 0.2% Triton X-100 and 5% goat serum.

After additional washes, rabbit anti-HIF-1α (Novus Biologicals; diluted 1:100 in PBS-Tween) was added for 60 min at room temperature followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (green; Invitrogen; diluted 1:500 in PBS-Tween) for 45 min at room temperature. After washing, samples were embedded in Prolong Gold antifade containing DAPI for counterstaining (blue; Invitrogen). Mounted samples were examined using an inverted confocal laser scanning 2-photon microscope (Olympus Fluoview FV1000, Olympus ×20/0.7 UPlanSApo objective) with Fluoview™ Spectral Scanning technology (Olympus America, Center Valley, Pa., USA) or, alternatively, using a Zeiss Axiolab microscope (Zeiss ×20/0.5 Plan-Neofluor objective; Carl Zeiss, San Diego, Calif., USA) with an attached Sony Digital Photo Camera DKC-5000 (Sony, USA) at calibrated magnifications. Mean fluorescence intensities as markers for HIF-1α expression levels were measured at equal exposure times and quantified using Image J (National Institutes of Health, Bethesda, Md., USA). HIF-1α levels were calculated by dividing the HIF-1α-specific signal by the background subtracted DAPI-related signal.

### *Mouse Infection Models*

Logarithmic phase GAS were resuspended in PBS and mixed 1:1 with sterile Cytodex beads (Sigma, St. Louis, Mo., USA), and an inoculum of  $5 \times 10^7$  CFU of GAS was injected subcutaneously into one flank of 10- to 12-week-old WT or TLR9-deficient mice (total injected volume per mouse 100 µl). The size of developing necrotic lesions was monitored daily for 4 days, at which time mice were euthanized and skin lesions were harvested for bacterial counts. In systemic challenge experiments, 12-week-old mice (WT and TLR9-deficient) were given intraperitoneal injections of mid-log phase GAS ( $5 \times 10^7$  CFU) in 500 µl of PBS or PBS alone as a control. After 24 h, blood was collected for bacterial counts.



**Fig. 1.** TLR9 is important for controlling GAS infection in vivo. **a** C57BL/6 (WT) and TLR9-deficient mice were injected subcutaneously with equivalent inocula of the M1 GAS strain, and skin lesion progression was measured for 4 days. **b** Representative photograph of skin lesions seen in the subcutaneous challenge experiment. **c** After 4 days, there were twice as many bacteria in the skin in the TLR9-deficient mice compared to the WT

mice. **d** Using an intraperitoneal infection model, a subset of the mice (5 WT and 4 TLR9-deficient mice) were bled 24 h after GAS infection, and bacteremia was evaluated by serial dilution of the blood. **e** Survival of WT ( $n = 13$ ) and TLR9-deficient ( $n = 12$ ) mice followed over 1 week after intraperitoneal infection. Data are presented as means  $\pm$  SEM. TLR9KO = TLR9-deficient.

### Statistics

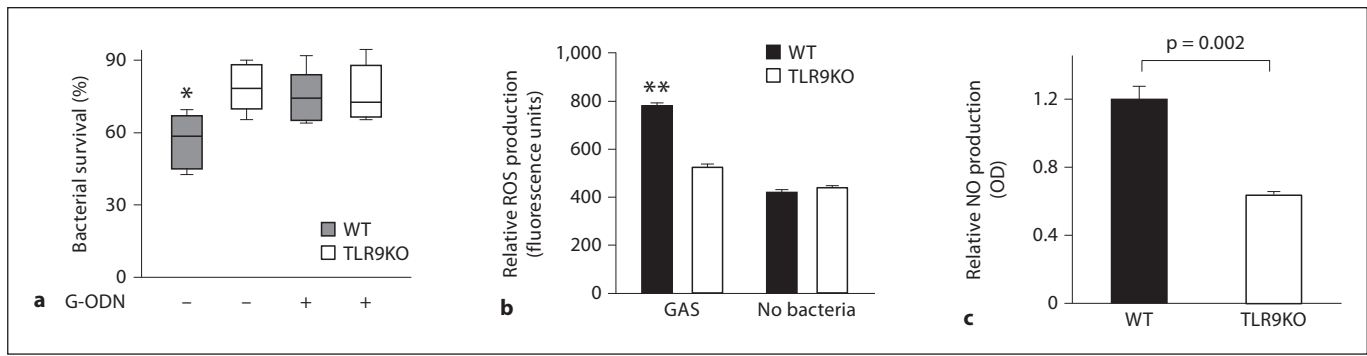
For comparisons of two groups of either paired or unpaired samples we used the nonparametric Wilcoxon test or t test, and for 3-sample comparison we used nonparametric Kruskal-Wallis one-way ANOVA on ranks. If the ANOVA analysis was significant, Bonferroni pair-wise group comparison was performed. The log rank test was used for survival analysis. p values were calculated using the software packages NCSS 2007 (NCSS, Kaysville, Utah, USA) and SPSS Statistics 17.0 (SPSS, Chicago, Ill., USA).

## Results and Discussion

To directly test the importance of TLR9 for the recognition and clearance of GAS in vivo, we challenged WT and TLR9-deficient mice with the invasive M1T1 GAS strain 5448 using a previously described model of necrotizing subcutaneous infection [25] and monitored the development of lesions over 4 days. TLR9-deficient mice de-

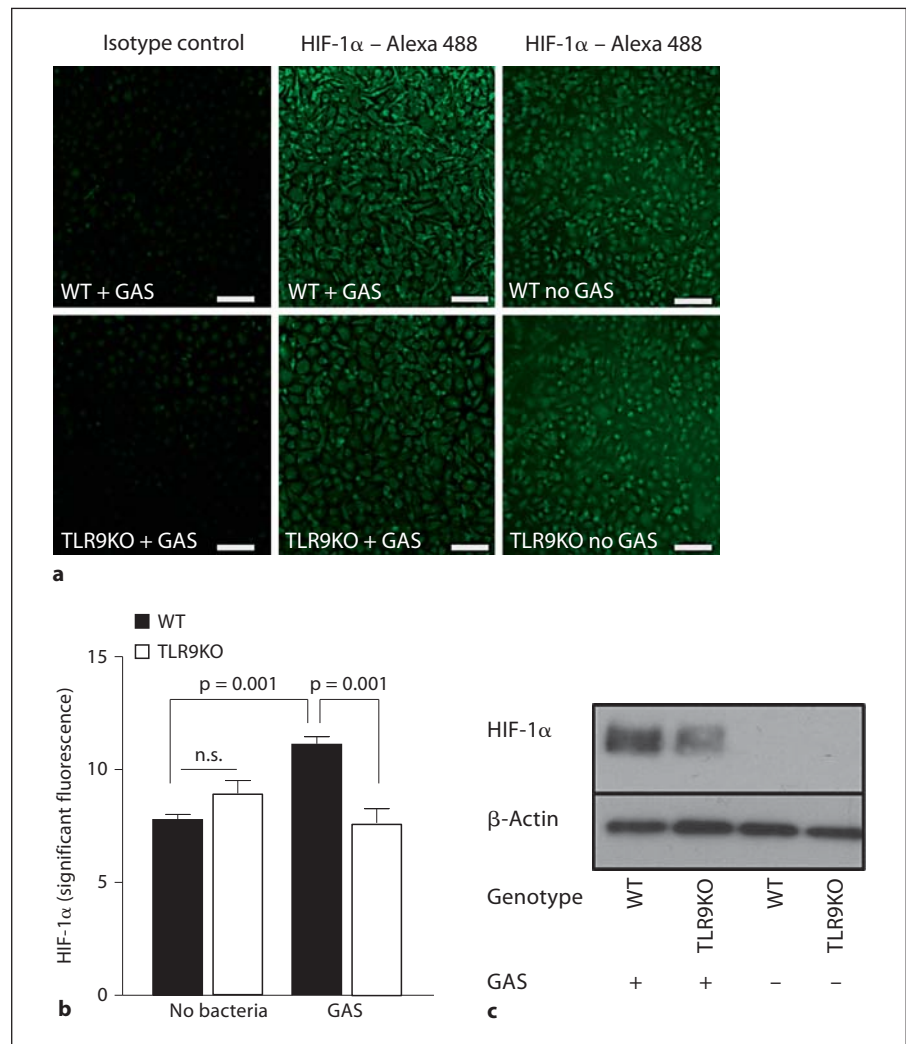
veloped significantly larger necrotizing skin lesions than did WT mice (fig. 1a, b). Mice were sacrificed on day 4, and the bacterial load in the excised skin lesions of TLR9-deficient mice was significantly higher than that in WT controls (fig. 1c).

GAS can cause bacteremic infections and toxic shock syndrome with high morbidity and mortality. To test the importance of TLR9 in a systemic infection model, WT and TLR9-deficient mice were injected intraperitoneally with logarithmic phase M1T1 GAS, and blood was collected at 24 h for enumeration of CFUs. TLR9-deficient mice resolved GAS bacteremia less effectively than did WT mice (fig. 1d), and the impaired GAS clearance observed in TLR9-deficient mice was reflected in increased mortality (fig. 1e). Altogether, these animal data show for the first time that TLR9 is important for controlling GAS in vivo during localized cutaneous or systemic infection.



**Fig. 2.** TLR9 mediates killing of GAS by macrophages. **a** Surviving GAS after incubation with WT macrophages or TLR9-deficient macrophages at an MOI of 1, with and without the TLR9 antagonist G-ODN. **b, c** WT and TLR9-deficient macrophages were incubated with GAS at an MOI of 1, and ROS (**b**) and NO (**c**) was quantified. Data are given as box plots with n = 6 per point

and 3 repeats (**a**) or as means  $\pm$  SEM with n = 6 (**b**) and n = 3 (**c**) (representative experiment from 3 independent experiments). ANOVA was significant at p = 0.01, with Bonferroni pair-wise group comparison at \* p < 0.05 and \*\* p < 0.005. TLR9KO = TLR9-deficient; OD = optical density at 540 nm.



**Fig. 3.** TLR9 promotes HIF-1α expression. WT and TLR9-deficient macrophages were challenged with GAS. **a** Representative immunofluorescence micrograph. **b** Quantification of HIF-1α expression (infected cells, n = 14, images of 3 independent experiments; noninfected cells, n = 8, images of 2 independent experiments). **c** Nuclear extracts of the same cells were analyzed for HIF-1α expression by Western blot with analysis of β-actin as a loading control. Data are presented as means  $\pm$  SEM. Scale bars = 100 μm. TLR9KO = TLR9-deficient; n.s. = not significant.



Macrophages, which express TLR9 [26], play a key role in the defense against GAS infections [27, 28]. We hypothesized that the impaired clearance of GAS in TLR9-deficient animals could be explained by inefficient killing of GAS by macrophages lacking TLR9 signaling. We thus studied the contribution of TLR9 to GAS clearance using in vitro killing assays and genetically or pharmacologically modified murine macrophages. GAS was killed efficiently when incubated with WT macrophages; however, killing was significantly impaired if TLR9 was absent or pharmacologically blocked by the TLR9 antagonist G-ODN (fig. 2a).

Macrophages kill GAS efficiently in part through production of ROS and NO [28]. Macrophages from WT and TLR9-deficient mice were incubated with GAS, and ROS and NO production was quantitated after 30 min. TLR9-deficient macrophages produced significantly less ROS (fig. 2b) and NO (fig. 2c) in response to GAS than did WT macrophages. These results suggest that impaired control of GAS infection in TLR9-deficient mice may in part be related to diminished NO- and ROS-mediated macrophage killing.

HIF-1 $\alpha$  is a key transcriptional regulator orchestrating myeloid cell inflammatory and innate immune responses [29]. GAS infection leads to increased levels of HIF-1 $\alpha$  in macrophages [23], and HIF-1 $\alpha$  is important for regulating macrophage production of ROS and NO in response to infection [23, 30]. We challenged WT and TLR9-deficient macrophages with GAS and determined the intranuclear content of HIF-1 $\alpha$  by immunostaining (fig. 3a, b; online suppl. fig. 1; for all online suppl. material, see [www.karger.com/doi/10.1159/000329550](http://www.karger.com/doi/10.1159/000329550)) and Western blotting (fig. 3c). After GAS challenge, HIF-1 $\alpha$  stabilization was less pronounced in TLR9-deficient compared to

WT mouse macrophages. The residual HIF-1 $\alpha$  induction still detectable in the TLR9-deficient mouse macrophages after GAS challenge could be due to the redundant effects of other pattern recognition receptors such as TLR2, TLR4 and TLR6, which contribute to HIF-1 $\alpha$  induction and stabilization [31, 32], likely through NF- $\kappa$ B-dependent increases in HIF-1 $\alpha$  transcript [33]. Also, GAS has been shown to be able to induce multiple inflammatory responses via MyD88-dependent TLR2/TLR4/TLR9-independent signaling [19], as has been shown for other Gram-positive pathogens [16, 34].

In summary, this study shows that TLR9 plays an important role in host defense against GAS infections and that stimulation of TLR9 improves macrophage killing of this leading human pathogen. TLR9-induced stabilization of the transcription factor HIF-1 $\alpha$  and increased generation of bactericidal ROS and NO are likely contributing factors to the observed innate immune phenotypes.

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## Disclosure Statement

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